REVIEW

State of Serum Markers for Detection of Cholangiocarcinoma

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Abstract

Difficulties in cholangiocarcinoma (CCA) management are largely due to the lack of specific biomarkers to diagnose CCA patients at an early stage. Most of CCA patients are diagnosed when the tumors have already extensively invaded and/or metastasized, resulting in poor survival. Definite diagnosis for CCA is through a histopathological study of e liver tissues, which is invasive to obtain the samples. The detection of CCA-associated markers in patients' sera seems to be a potential diagnostic approach, which is less invasive, inexpensive, and does not require a specialist to perform the diagnosis. To date, several serum markers, such as carcinoembryonic antigen, carbohydrate antigen (CA) 19-9, CA242, CCA-associated carbohydrate antigen, mucin glycoproteins, cytokines, etc., have been reported to be the potent diagnostic and prognostic markers for CCA. However, a single marker determination exhibits low sensitivity and specificity for diagnosis of CCA. Alternatively, multiple marker analysis seems to have more optimistic prospects for improvement of diagnostic and prognostic determination of CCA.

Keywords: Tumor - diagnosis - prognosis - bile duct - early diagnosis - blood

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Introduction

Cholangiocarcinoma (CCA) is rare in western countries but is one of the major public health problems in northeast Thailand due to its high incidence, severity and high mortality rate (Patel, 2001; Endo et al., 2008; Shin et al., 2010). At present, there are several advanced techniques for demonstrating CCA, e.g., ultrasonography and CT scanning for detection of dilated intrahepatic biliary ducts; cytology and brushings done at the time of percutaneous transhepatic cholangiography or endoscopic retrograde cholangiography to demonstrate tumor cells in bile; and fine needle aspiration to identify tumors in jaundiced patients. However, the late detection and poor survival after diagnosis has led to a need for more powerful markers or techniques for early diagnosis of CCA. Complete resection provides the best hope for long-term survival, but the difficulty in establishing preoperative diagnosis of CCA limits the number of successful treatments (Guglielmi et al., 2009; Morise et al., 2010).

There are several types of biomarkers for cancer and each provides different benefits based on the stage of disease identified (Figure 1). Risk biomarkers are to identify the person who has risk of developing cancer with no measurable disease whereas early detection involves a high-risk population, a screening test, and a testing schedule (Hassanein et al., 2012). It distinguishes populations of individuals at-risk before or after the disease becomes measurable. There are several markers suggested to diagnose cancer. Diagnostic biomarkers are used to determine whether cancer is present with measurable asymptomatic disease and prognostic biomarkers usually identify individuals with an aggressive phenotype and shorter survival. It is constructive information for clinicians to select the choice of treatment that is appropriate and of benefit for individual patients (Hassanein, et al., 2012). Herein, we review recent reports in the field of biomarkers for risk assessment and diagnosis of *Opisthorchis viverrini* (Ov)-associated CCA. The clinical use and limitations of different approaches will be discussed. The markers for non-liver fluke related CCA is beyond the scope of this review.

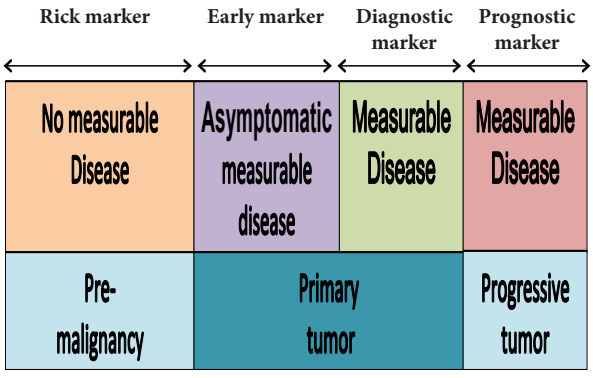

Figure 1. Biomarker Provides Different Benefits Based on the Stage of Disease Identified

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Risk and Early Markers for CCA

There are several factors identified as risks for CCA. It is well documented that liver fluke, Ov, is the strong risk factor of CCA found in Southeast Asia, especially in northeast Thailand (Haswell-Elkins et al., 1994; Sithithaworn and Haswell-Elkins, 2003; Bouvard et al., 2009). Subsequently, infection of the related liver fluke *Clonochis sinensis* has been acknowledged to be the risk factor of CCA in south China, Korea and Vietnam (Khan SA, e tal., 2008). For CCA diagnosed in Western countries, several risk factors are listed, e.g., primary sclerosing cholangitis, hepatolithiasis, choledochal cyst, caroli's disease, cirrhosis, hepatitis C, and toxins (dioxin, polyvinyl chloride) etc. (Charbel and Al-Kawas, 2011). Even though different factors are raised as risk determinants of CCA from different parts of the world, most of these factors contribute to chronic inflammation and chronic injury of the biliary epithelium as common features associated with CCA.

Many factors have been raised to be the risk markers for Ov-associated CCA e.g., acute or past history of Ov infection (Srivatanakul et al., 1991; Pinlaor et al., 2004), high level of serum anti-Ov antibody (Akai et al., 1994; Pinlaor et al., 2012; Sawangsoda et al., 2012), and periportal fibrosis detected by ultrasonography (Mairiang et al., 2006; 2012). Clinicians and researchers believe that the opportunity for improved survival increases if cancer is diagnosed in its early stage. The use of chest computed tomographic (CT) imaging drastically improved the detection rate and survival of early-stage lung cancers (Henschke et al., 1999). A large randomized screening study of lung cancer by low-dose chest CT resulted in an improvement in overall survival and a 20% reduction in lung cancer–specific mortality (Aberle et al., 2011). This impressive result gives new hope of improvement in overall survival of many cancers.

Not only for CCA but for all malignancies, surgeons as well as scientists believe that early detection of cancer can increase the curative rate and improve survival of patients. However, CCA is still one of the most difficult cancers to diagnose at early stage since there are no specific symptoms when tumor develops. In addition, CCA is a heterogeneous tumor with the complexity of the genome and the proteome. Many researchers are taking up the challeng to bring specific biomarker candidates from the bench to the bedside.

There are a number of serum markers for detection of CCA (Table 1); for example, carcinoembryonic antigen (CEA), carbohydrate antigen (CA) 19-9, and serum mucin (Wongkham et al., 2003; Bamrungphon et al., 2007; Briggs et al., 2009; Silsirivanit et al., 2011). However, the uncertainties of their overall accuracy limit the use of these markers in early detection. In general, these markers are useful for identifying and managing patients but are not appropriate for screening and early diagnosis because the detection of these markers in blood often signifies the presence of an advanced tumor (Bjornsson et al., 1999; Carpelan-Holmstrom et al., 2002; Qin et al., 2004; Alvaro, 2009). Moreover, patients without cancer may harbor low levels of these markers in blood (Szekanecz et al., 2007; 2008; Bergamaschi et al., 2012). This limitation has led to specific cut-off levels being set up based on population studies of cancer patients and controls to identify the lower limits of a positive test.

The major factor that limits the finding of early marker is the limitation to access early-stage tumor tissue samples for definite diagnosis and the low abundance of potential biomarkers in bio-fluids. At present, there is no early marker for identifying CCA. To ascertain whether the marker candidate is the risk marker, there must be a screening trial for high risk CCA to follow the cohort until the disease appears. Since CCA frequently develops in patients with any of a variety of preexisting bile duct diseases, some of them are considered precursors of CCA (e.g., biliary lithiasis, clonorchiasis, opisthorchiasis, and primary sclerosing cholangitis), a long term surveillance of these high risk populations with testing schedule will reveal the marker candidates that may not only predict CCA in individuals but also diagnose CCA in an early stage.

Current Status of Serum Markers for Cholangiocarcinoma

The availability of a rapid and formal proof of malignancy by using less invasive procedures is a teleological goal of the diagnosis of CCA. Although cytology is the most specific routine diagnostic procedure, its sensitivity is insufficient at around 50-60%, and thus diagnosis is usually carried out by more invasive techniques such as liver biopsy. Serum marker is an attractive detection strategy for screening, due to their ease of acquisition and noninvasive access to large quantities of samples available for analysis.

The development of CCA is frequently associated with high blood levels of tumor markers such as CEA, CA19-9, cytokeratin (CK) 19, interleukin 6, and mucin MUC5AC (Civardi et al., 1986; Ramage et al., 1995; Goydos et al., 1998; Patel et al., 2000; Boonla et al., 2003; Wongkham et al., 2003; Qin et al., 2004; Tangkijvanich et al., 2004; Bamrungphon et al., 2007; Sripa et al., 2012). Among them, CEA and CA19-9 are the most commonly studied and routinely used markers. Nevertheless, in almost all series on serum CEA, high false-positive results are mentioned (Fisher et al., 1995; Bjornsson et al., 1999; Bettinardi et al., 2003; Qin et al., 2004; Ni et al., 2005; Li and Zhang, 2009). A good serum marker should exhibit high diagnostic values, e.g., sensitivity, specificity, positive and negative predictive values and accuracy (Figure 2). As for the CCA markers, they should, thus,

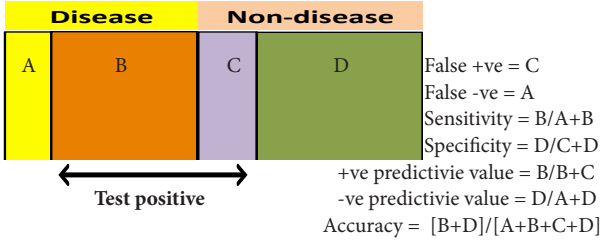

Figure 2. Several Indices Indicate the Diagnostic Value of Each Test

Table 1. The Diagnostic Marker Detected in Serum for CCA

Serum markers	Sensitivity (%)	Specificity (%)	Cases and control used in the studies	References
BALP	65	88	77 CCA, 55 GI-CA, 18 BLD, 30 HE	(Bhudhisawasdi et al., 2004)
CA19-9 (cut off 100 U/ml)	53-79	81-99	139 CCA, 384 BLD, 66 HE	(Ramage et al., 1995; Patel et al., 2000; Qin et al., 2004; Levy et al., 2005; Leelawat et al., 2006)
CCA-CA	88	90	97 CCA, 47 GI-CA, 43 BLD, 52 OV, 51 HE	(Silsirivanit et al., 2011)
CEA (cut off 22 ug/ml)	53-69	82-86	50 CCA, 114 BLD, 15 HE	(Ramage et al., 1995; Qin et al., 2004)
CYFRA 21-1	75-87	92-95	94 CCA, 164 HCC, 177 BLD	(Uenishi et al., 2003; Uenishi et al., 2008)
hTERT mRNA	85	78	39 CCA, 50 BLD, 10 HE	(Leelawat et.al., 2006)
IL-6	71-80	26-98	207 CCA, 55 HCC, 41 LMC, 225 BLD, 299 HE	(Goydos et al., 1998; Tangkijvanich et.al., 2004; Cheon et.al., 2007; Sripa et.al., 2012)
M2-PK	84	90	115 CCA, 85 BLD, 120 HE	(Li and Zhang, 2009)
MMP7	75-76	46-78	103 CCA, 164 BLD	(Leelawat et al., 2009; Leelawat et al., 2010; Prokobwong et al., 2012)
MUC5AC	63-71	90-97	348 CCA, 90 GI-CA, 92 BLD, 60 OV, 104 HE	(Wongkham et al., 2003; Bamrungphon et al., 2007)
NGAL (cut off 110 ng/ml)	76	72	50 CCA, 50 BLD	(Leelawat et al., 2011)
P53	100	39	7 CCA, 151 GI-CA	(Attallah et.al., 2003)
Periostin	88	89	8 CCA, 24 HCC, 13 LMC, 26 BLD	(Fujimoto et al., 2011)
RCAS1 (cut off 10 U/ml)	74	96	23 CCA, 20 HCC, 52 BLD, 35 HE	(Watanabe et al., 2003)
TSA	72-83	58-86	158 HCC, 135 BLD, 93 HE	(Wongkham et al., 2001; Kongtawelert et al., 2003)

*BALP, biliary alkaline phosphatase; CA19-9, carbohydrate antigen19-9; CEA, carcinoembryonic antigen; CCA-CA, cholangiocarcinoma associated carbohydrate antigen; carcinoembryonic antigen; CYFRA 21-1, cytokeratine 19 fracment 21-1; hTERT, human telomerase reverse transcriptase; Il-6, interleukin-6; M2-PK, M2-pyruvate kinase; MMP7, matrix metalloproteinase 7; MUC5AC, mucin 5AC; NGAL, neutrophil gelatinase-associated lipocalin; RCAS1, receptor binding cancer antigen expressed in SiSO cells, TSA, total sialic acid; GI-CA, other gastro intestinal cancers; BLD, benign liver diseases, HE, healthy persons; OV, opisthorchiasis; HCC, hepatocellular carcinoma; LMC, lievr metastatic cancers

Table 2. The Prognostic Marker Detected in Serum for CCA

Serum amrker	Clinical correlation	References
CA19-9 > 100 U/ml		
	Poor prognosis	(Huang et al., 2004; Heimbach
	Poor survival	et al., 2006; Briggs et al., 2009)
CCA-CA	Tumor berden	(Silsirivanit et al., 2011)
	Poor survival	
CYFRA21-1 > 2.7 ng/ml		
	Tumor size and number	(Uenishi et al., 2008)
	Lymphatic invasion	
	Poor prognosis	
	Poor survival	
IL-6	Tumor berden	(Cheon et al., 2007)
MUC5AC	Tumor size	(Boonla et al., 2003; Bamrungphon
	TNM stage IVA-B	et al., 2007; Matull et al., 2008)
	Poor survival	
RCAS1	Poor survival	(Enjoji et al., 2004)
	Tumor berden	

*CA19-9, carbohydrate antigen 19-9; CCA-CA, cholangiocarcinoma associated carbohydrate antigen; carcinoembryonic antigen; CYFRA 21-1, cytokeratine 19 fracment 21-1; Il-6, interleukin-6; MUC5AC, mucin 5AC; NGAL, RCAS1, receptor binding cancer antigen expressed in SiSO cells

Table 3. Combinatorial Serum Markers for CCA Diagnosis

Markers	Sensitivity (%)	Specificity (%)	References
CA19-9	60	91	(Ramage et al., 1995)
CEA	53	86	
CEA -CA19-9 Index	66	100	
CA19-9	66	88	(Qin et al., 2004)
CEA	69	82	
CEA or CA19-9	91	76	
CEA and CA19-9	63	87	
CEA	28	85	(Ni et al., 2005)
CA19-9	73	59	
CA242	28	86	
CEA and CA19-9	21	90	
CEA and CA242	9	95	
CA19-9 and CA242	26	91	
CEA and CA19-9 and CA242	9	95	

*CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; CA242, carbohydrate antigen 242

differentiate CCA from benign biliary tract diseases.

Some tumor markers in serum showed the association with the clinical outcome of CCA patients (Table 2), and those serum markers may possibly be used for monitoring and prognostic prediction of CCA. Several tumor markers have been detected in clinical samples, such as tissues, bile, serum and plasma, obtained from CCA patients. However, the most practical aspect is the detection of tumor markers in patient serum.

This review, therefore, will focus on the currently available serum markers which are continuously reported to be the diagnostic and prognostic markers for CCA.

Carbohydrate Antigens and Glycoproteins

Several carbohydrate antigens and glycoproteins have been reported to be CCA-associated markers: CEA, CA19-9, CA125, etc., and of these CA19-9 is the most frequently reported marker for CCA.

Carcinoembryonic antigen (CEA)

CEA is a 200 kDa glycoprotein that has been shown by immunohistochemical methods to be present in malignancies of the colon, pancreas, and biliary tree (Goslin et al., 1981; Kojima et al., 1984; Nonomura et al., 1987; Albers et al., 1988; Ichihara et al., 1988). The incidence of abnormal serum levels of CEA in hepatolithiasis associated with CCA was higher than in hepatolithiasis patients without CCA. Undiagnosed CCA was suggested to be in hepatolithiasis patients with high level of serum CEA (Sasaki et al., 1996; Chen et al., 2002).

Carbohydrate antigen (CA) 19-9

CA19-9 is a glycosphingolipid of the Lewis blood

group that for years has been proposed as a useful marker for epithelial type gastrointestinal cancers, especially of the pancreas and biliary tract (Chen et al., 2002; Qin et al., 2004; Ni et al., 2005). CA19-9 is a ligand of E-selectin that may play a role in the adhesion of cancer cells to endothelial cells, resulting in hematogenic metastasis. The CA19-9 antigen in tissues exists primarily as an epitope present on a glycolipid, sialo-lacto-N fucopentaose II ganglioside, but it is associated with mucin in serum (Tempero et al., 1989; Robinson et al., 1993; Yue et al., 2011). The oligosaccharide on which the CA19-9 epitope is found is a sialylated Lewis A blood group antigen (Magnani et al., 1982).

The CA19-9 is a tumor-associated, but not a tumor-specific, antigen. It is synthesized by normal human pancreatic and biliary ductular cells, as well as by gastric and colonic epithelial cells (Dietel et al., 1986; Imamura et al., 1990; Yamauchi et al., 1993). It is present in large quantities in normal pancreatic juice (Tocchi et al., 1998) and in the bile of patients with benign disorders (Albert et al., 1988; Ohshio et al., 1990).

Serum CA19-9 is commonly used as a tumor marker for gastrointestinal malignancies, especially for the diagnosis of pancreatic carcinoma. Serum CA19-9 was first introduced to differentiate CCA from benign biliary tract diseases with 73% sensitivity (Jalanko et al., 1984). Patients with CCA frequently show high CA19-9 serum level (57-79%) (Ramage et al., 1995; Patel et al., 2000; Qin et al., 2004; Levy et al., 2005; Leelawat et al., 2006). Using mean + 3SD as the cut-off level, 57.1% of CCA patients had elevated CA19-9 and 28.6% had elevated CA125 (Pungpak et al., 1991). In patients with clinically suspected CCA, 71.4% had elevated CA19-9 and 28.6% had elevated CA125. The measurement of CA125 and CA19-9 may be useful in the early detection of opithorchiasis associated CCA. As high CA19-9 serum level is not completely specific for cancer, it was suggested to be a valuable adjunct marker for CCA.

It is well known that moderate increase in plasma concentration of CA19-9 was found in 15-36% of patients with benign pancreatic, liver and biliary tract diseases (Albert et al., 1988; Ohshio et al., 1990; Morris-Stiff et al., 2009). High CA19-9 levels were also documented in several benign biliary tract diseases, such as lithiasis (Albert et al., 1988), autoimmune cholangitis (Maestranzi et al., 1998) and acute cholangitis (Steinberg, 1990; Ker et al., 1991). The increased production of CA19-9 from the biliary epithelial cells and the decreased hepatobiliary clearance due to cholestasis may have contributed to abnormal CA19-9 elevation in the blood stream (Albert et al., 1988; Ker et al., 1991; Ng et al., 1995; Singh et al., 2011).

Whether CA19-9 should be used in the clinical diagnostic work-up of patients with biliary tract diseases still remains a difficult question to answer. Extremely high and continuously increasing CA19-9 concentration, may implicate neoplasia. The biochemical detection of CA19-9 has never been regarded as a gold standard but rather as a helpful indicator when searching for biliary malignancy. Medical history, clinical examination, qualitative radiology methods, and careful follow-up are the hallmarks helpful for diagnosing an extremely high serum CA19-9 value cases.

Cabohydrate antigen 125 (CA125)

CA125 is the glycan of mucin16 (MUC16) which was first reported as a biomarker for the diagnosis of ovarian cancer (Scholler and Urban, 2007). The exact molecular structure of CA125 is still unclear (Weiland et al., 2012). Similar to many tumor markers, the detection of serum CA125 is commercially available as a simple ELISA kit (Scholler and Urban, 2007). The elevated CA125 in sera was found in 24-47% of CCA patients. The level of CA125 reflected the presence of CCA as serum CA125 level decreased after tumor resection (Pungpak et al., 1991; Su et al., 1996; Chen et al., 2002) and increased with tumor recurrence (Chen et al., 2002).

Cabohydrate antigen 242 (CA242)

CA242, a carbohydrate antigen detected by monoclonal antibody C242, has been used as a tumor marker for pancreatic and colon cancers (Lindholm et al., 1983; Nilsson et al., 1992; Ni et al., 2005; Yang et al., 2011; Yang et al., 2012). The structure of CA242 is not completely defined, but the presence of sialiated-type I chain carbohydrate moiety was suggested (Nilsson et al., 1992). Using an electrochemiluminescence immunoassay, serum CA242 level was found to be elevated in CCA and pancreatic cancer with 68% and 63% sensitivity, respectively (Ni et al., 2005). As serum CA242 level of CCA patients was significantly higher than those of hepatoma, serum CA242 can therefore be used to distinguish CCA from hepatoma patients (Tao et al., 2010).

Cholangiocarcinoma-associated carbohydrate antigen (CCA-CA) or S121

Recently, A S121 mAb recognizing a novel glycan epitope was established (Silsirivanit et al., 2011). The results of S121-immunohistochemistry of CCA tissues indicated that S121 reactive antigen was present in hyperplastic/dysplastic and neoplastic bile ducts but not in normal bile duct epithelia, and the antigen was designated as cholangiocarcinoma-associated carbohydrate antigen (CCA-CA). A sandwich ELISA, using soybean agglutinin and S121 mAb, was developed for detecting CCA-CA in patients' sera. The CCA-CA level in the sera of CCA patients was significantly higher than those of healthy controls (healthy persons, active Ov infected persons) or patients of other gastrointestinal diseases (gastric cancers, hepatoma, and benign hepatobiliary diseases), with 87.63% sensitivity, 89.58% specificity, 80.95% and 93.47% for positive and negative predictive values. High levels of serum CCA-CA was related with poor outcome of patients and possibly could be a prognostic marker of CCA. A study of Ov-associated CCA in a hamster model revealed that CCA-CA was elevated in biliary epithelium at the early stage of carcinogenesis, suggesting the possibility of CCA-CA as the early marker for CCA (Sawanyawisuth et al., 2012).

Mucins

Mucins (MUC) are a group of high molecular weight

O-linked glycoproteins which have been reported to be increased in the sera of CCA patients and MUC1, MUC4, MUC5AC and MUC6 are considered as the potential diagnostic and prognostic markers for CCA (Boonla et al., 2003; Wongkham et al., 2003; Shibahara et al., 2004; Boonla et al., 2005; Tamada et al., 2006; Bamrungphon et al., 2007; Matull et al., 2008; Thuwajit et al., 2008; Xu et al., 2008; Alvaro, 2009; Silsirivanit et al., 2011). The most emphasized mucin in CCA is MUC5AC, which was detected in both tumor tissues and sera of the patients. MUC5AC mucin in sera was first qualitatively measured for diagnosing CCA using agarose gel electrophoresis (Wongkham et al., 2003; Bamrungphon et al., 2007). Later, a sandwich ELISA using anti-MUC5AC mucin monoclonal antibody and soybean agglutinin was developed for quantitative determination of serum MUC5AC (Wongkham et al., 2003; Bamrungphon et al., 2007). The assay at the cut-off value of absorbance 0.074 could discriminate CCA patients from the controls with 71% sensitivity and 90% specificity. Serum MUC5AC in CCA patients was significantly higher than that in patients having other gastrointestinal cancers, benign liver diseases, opisthorchiasis, and healthy persons. The test is simple to perform, reproducible, and probably used for detecting CCA in a high-risk group or suspected patients.

Besides MUC5AC, sialylated carbohydrate antigen KL-6 of MUC1 detected by ELISA in the sera of CCA patients was also higher than those of healthy persons, patients with hepatoma and other liver metastatic cancers (Xu et al., 2008). Using immunohistochemisitry, high expression of biliary MUC1 was shown to distinguish CCA patients from those of benign liver diseases with 90% sensitivity and 76% specificity (Matsuda et al., 2010). Other mucins such as MUC4 and MUC16 were also overexpressed in CCA tissues and related to poor survival of CCA patients (Tamada et al., 2006; Matull et al., 2008; Yeh et al., 2009; Higashi et al., 2012).

Total sialic acid (TSA)

Serum TSA levels of CCA patients measured by periodate-resorcinaol microassay or thiobarbituric acid assay were higher than those of hepatoma patients, benign liver diseases, and healthy control with 72-83% sensitivity and 58-86% specificity (Wongkham et al., 2001; Kongtawelert et al., 2003). However, the correlation between serum TSA level and clinical parameters of CCA patients was not clear.

Enzymes and Isoenzymes

A number of serum enzymes were elevated in CCA patients' sera and are possibly used as biomarkers for CCA, e.g., biliay alkaline phosphatase, metalloproteinases, trypsinogen, telomerase, and private kinase.

Biliary alkaline phosphatase (BALP)

Isoenzymes, or isozymes, are different molecular species of enzymes catalyzing the same reaction. Cancer tissue frequently expresses altered isozyme patterns, reflecting altered metabolism of the tumor. At least four alkaline phosphatase (ALP) isozymes encoded by different genetic loci were found in human serum: tissue-nonspecific found in liver, bone and kidney; intestine; placenta and placenta-like or germ cell. Biliary alkaline phosphatase (BALP), an isoform of liver-ALP, has been found in the sera of patients with biliary obstruction and metastatic liver cancer. We have compared BALP levels in the sera of patients with CCA and those of non-jaundiced benign hepatobiliary diseases, other cancers and healthy person. BALP isoform was detected in 65% of CCA patients independently to jaundice condition or histological grading of the tumor (Bhudhisawasdi et al., 2004). The serum BALP level in non-jaundiced CCA was significantly lower than that of jaundiced CCA, but not correlated with serum bilirubin level. Of note, however, was that no BALP was detected in the sera of healthy persons. In the patients with high serum ALP (>147U/L), BALP can differentiate non-jaundiced CCA patients from other non-jaundiced carcinoma patients with 85% sensitivity, 79% specificity, positive and negative predictive values of 81% and 83%, respectively (Bhudhisawasdi et al., 2004). The determination of BALP isozymes is useful to detect non-jaundiced CCA patients and progressive disease in CCA.

Matrix metalloproteinases 7 (MMP7)

There are more than 25 structurally related matrix metalloproteinase family, zinc-dependent, endopeptidases that can degrade various components of the extracellular matrix (ECM) (Woessner, 1994). They are involved in ECM remodelling, which are essential in several physiologic processes – such as wound healing, bone resorption, organogenesis –as well as pathologic conditions including inflammatory, vascular and autoimmune disorders, carcinogenesis and tumor metastasis (Kahari and Saarialho-Kere, 1999; Egeblad and Werb, 2002). Proteolytic degradation of the ECM alters the cell–cell and cell–ECM interactions which are essential for tumor invasion and metastasis (Liotta et al., 1980; Folkman, 1995; Kleiner and Stetler-Stevenson, 1999). In this regard, MMPs were upregulated in clinical specimens of virtually all human malignancies (Nawrocki et al., 1997; Gonzalez-Avila et al., 1998; Kugler et al., 1998; Sutinen et al., 1998). Overexpression of various MMPs was strongly associated with the invasive behavior of tumor cells and their metastatic ability in experimental animal models (Bernhard et al., 1994; Tsunezuka et al., 1996). It has been demonstrated that MMPs contribute to the matrix-degrading activities in metastasis process as well as proteolytic activity on several non-matrix substrates such as chemokines, adhesion molecules, growth factors, growth factor receptors, pro-apoptotic and anti-apoptotic molecules (McQuibban et al., 2002; Hemers et al., 2005) in tumor progression. MMP1, MMP2 and MMP7 exert primarily cancer-promoting effects, whereas MMP3, MMP8, MMP9, MMP12 and MMP14 have been documented to exert anti-cancer effects. MMP7 was reported to be elevated in serum and associated with poor survival of the patients in many types of cancers, such as colorectal, gastric, ovary, and CCA (Leelawat et al., 2009; Szarvas et al., 2010; Yeh et al., 2010; Zohny and Fayed, 2010; Garcia-Albeniz et al., 2011). In CCA, MMP7 was

reported to be elevated in both tissues and sera of CCA patients (Miwa et al., 2002; Itatsu et al., 2008; Leelawat et al., 2009; Hirashita et al., 2012; Prakobwong et al., 2012). Plasma and serum MMP7 levels were significantly higher in CCA patients than those in healthy persons and benign liver diseases patients with 69-75% sensitivity and 72-78% specificity (Leelawat et al., 2009; Leelawat et al., 2010; Prakobwong et al., 2012). Moreover, the high level of MMP7 was correlated with metastasis and poor survival of CCA patients (Miwa et al., 2002; Itatsu et al., 2008; Hirashita et al., 2012).

M2-pyruvate kinase (M2-PK)

M2-PK is an enzyme in the glycolysis pathway. It was markedly elevated in the sera of CCA patients with 84.2% sensitivity and 90% specificity in discriminating CCA from benign liver diseases and healthy controls (Li and Zhang, 2009).

Serum trypsinogen-2 and trypsin-2-α-antitrypsin

Trypsinogen-2, a 25 kD isozyme of trypsinogen, is an inactive precursor of trypsin secreted in pancreatic fluids. Trypsin-2-α-antitrypsin or α1-antitrypsin, a single-chain glycoprotein consisting of 394 amino acids, is a trypsin inhibitor of serpin superfamily. High concentrations of serum trypsinogen-2 (>90 μg/l) and trypsin-2-α-antitrypsin (>25 μg/l) detected by quantitative immunofluorometric assay were found most often in patients with biliary and pancreatic cancer, as well as in benign obstructive biliary disease. The validity of these tests for detection of CCA in patients was 46% and 42%, respectively (Hedstrom et al., 1996a; Lempinen et al., 2007). In addition, the elevation of trypsinogen-2 and trypsin-2-α-antitrypsin in serum and urine were reported in patients with severe acute pancreatitis (Hedstrom et al., 1996b; Kemppainen et al., 1997; Hedstrom et al., 2001; Lempinen et al., 2003).

Human telomerase reverse transcriptase (hTERT)

hTERT, a catalytic subunit of the enzyme telomerase is a ribonucleoprotein polymerase that maintains telomere ends (Kirkpatrick and Mokbel, 2001) and allows senescent cells that would otherwise undergo apoptosis to become potentially immortal which is often occurred in cancerous cells. Deregulation of telomerase expression in somatic cells may be involved in oncogenesis. hTERT-mRNA was frequently (84.9%) detected in the sera of CCA patients but was low (21.9%) in benign liver diseases, and not detected in the sera of healthy subjects (Leelawat et al., 2006).

Others

Cytokeratin 19

The imunohistochemical studies of cytokeratin (CK) on surgically resected liver tumors indicated that of 30 CCA tissues, 97% were positive for CK7, 77% for CK19 and CK23, while most of HCC and metastatic colorectal adenocarcinoma showed negative staining for these CKs (Maeda et al., 1996). Therefore, these markers are considered to be useful not only as the diagnostic criteria but also for the differential diagnosis of primary and metastatic carcinomas of the liver. CKs can be detected in serum. Levels of serum CYFRA21-1, a fragment of CK19, exceeded 9 ng/ml was reported for CCA patients. Marked increase in serum CYFRA21-1 levels in patients with intrahepatic CCA particularly in those with normal levels of α-fetoprotein, would suggest the existence of intrahepatic CCA rather than HCC. In addition, high level of serum CYFRA21-1 was associated with poor prognosis of CCA patients (Uenishi et al., 2003; Uenishi et al., 2008).

Interleukin-6 (IL-6)

IL-6 is a growth factor for bile duct epithelium. HuCC-T1, a human CCA cell line, expressed IL-6 mRNA and secreted a large amount of biologically active IL-6 in the culture medium (Okada et al., 1994). The cell growth was significantly inhibited in the presence of anti-human IL-6 antibody in the culture medium. These findings indicate that IL-6 is one of the autocrine growth factors of CCA cell line in vitro.

High levels of serum IL-6 have been shown to signify an adverse prognosis in many cancer patients, including colorectal, gastric and prostate cancers, etc. Not only in cancer, serum IL-6 is also elevated in several physiological and pathological conditions such as aging, infection and inflammation (Nakashima et al., 2000; Kim et al., 2008; Naugler and Karin, 2008). By exclusion of patients who have high serum IL-6 level with known causes, such as hepatoma and liver-metastatic colorectal cancer, serum IL-6 level could identify patients with CCA and whether they were correlated with tumor burden both before and after resection (Goydos et al., 1998; Sirica, 2005; Cheon et al., 2007; Mott and Gores, 2007; Allison et al., 2009; Alvaro, 2009). Serum IL-6 level can differentiate CCA patients from healthy persons and patients of other hepatobiliary diseases with 71-80% sensitivity but with a divert specificity (26-98%) depending on the control group included in each study (Goydos et al., 1998; Tangkijvanich et al., 2004; Cheon et al., 2007; Sripa et al., 2012) . The level of plasma IL-6 was reported to be elevated in opisthorchiasis patients with advanced periductal fibrosis and at a higher level in CCA patients, compared with those of healthy persons and opisthorchiasis patients without advanced periductal fibrosis (Sripa et al., 2012). Serum IL6 with advanced periductal fibrosis may be useful as combination markers to indentify the people with high risk of CCA.

Neutrophil gelatinase-associated lipocalin (NGAL)

NGAL, a 25 kDa glycoprotein in the lipocalin super-family, in serum can be determined by ELISA method. Serum NGAL level in the sera of CCA patients was significantly higher than that of patients with benign liver diseases. The diagnostic sensitivity and specificity of serum NGAL to discriminate CCA from benign liver diseases was 76% and 72%, respectively (Leelawat et al., 2011).

Periostin

Periostin is an extracellular matrix protein belonging to the fasciclin family. Elevation of serum periostin in CCA was reported, with 88% sensitivity and 89% specificity, to

distinguish CCA patients from other hepatic malignancies and healthy people (Fujimoto et al., 2011).

p53 protein

p53 protein was detected in 100% of serum samples of CCA patients (n =7). However, the specificity was low, since P53 protein was also detected in many cancers, such as, carcinoma of pancreas, colon, gastric, and etc (Attallah et al., 2003).

Receptor-binding cancer antigen expressed in SiSo cells (RCAS1)

RCAS1 is a tumor-associated antigen detected by monoclonal antibody 22-1-1 raised against the human uterine carcinoma cell line SiSO. The serum RCAS1 level can be determined by ELISA. Serum RCAS1 was reported to be elevated in various types of cancer including CCA, and a few cases of benign liver diseases (Watanabe et al., 2003; Enjoji et al., 2005). Serum RCAS1 in CCA patients was higher than those of healthy persons and patients with other hepatobiliary diseases with 74% sensitivity (Watanabe et al., 2003; Enjoji et al., 2005). Moreover, serum RCAS1 level was associated with tumor burden, with a significant reduction after surgical treatment and increase with recurrence of CCA (Enjoji et al., 2004).

Serum α1β-glycoprotein and afamin ratio

We recently validated a serum-based proteomic signature for CCA diagnosis. We analyzed low abundant proteins in sera of CCA patients in comparison with healthy subjects using two-dimensional polyacrylamide gel electrophoresis (Tolek et al., 2012). Of the 36 proteins identified as CCA associated proteins, α1β-glycoprotein (A1BG) and afamin (AFM) were detected consistently at different degrees in CCA sera compared to controls. A single blot test of traditional SDS-polyacrylamide gel electrophoresis was developed to assess the A1BG/AFM ratio in the sera. The test could detect CCA cases with 87.5% specificity and 84.4% sensitivity. The levels of A1BG/AFM ratio were significantly higher in CCA cases compared to controls. In addition, high level of post-operative serum A1BG/AFM ratio was associated with worse outcomes and the infiltration of resection margins. The use of the A1BG/AFM ratio as a marker for monitoring CCA during treatment and/or detection of recurrent CCA was proposed. However, prospective studies are awaited to demonstrate the clinical value of this observation.

Combined Markers

Diagnosis and/or prognosis of malignant tumors by a single marker are often not satisfactory in clinical practice. The simultaneous measurement of multiple markers often provides a more precise diagnosis or prognosis than a single marker. In particular, a combination of multiple markers is thought to increase greatly the sensitivity and specificity for cancer detection. Evaluation of diagnosis/prognosis values for CCA by using multiple markers are summarized in Table 3. The combined analysis of serum CA19-9 and CEA improved the diagnostic values compared to the use of individual markers (Ramage et al., 1995; Qin et al., 2004). An index of using the formula CA19-9 +(CEA x 40) gave an accuracy of 86% in diagnosis of CCA (Ramage et al., 1995). On the other hand, the combination of CEA, CA19-9 and CA242 improved the specificity of discriminating CCA patients from patients with benign and malignant pancreatic diseases up to 95%, however the sensitivity decreased to 9% (Ni et al., 2005).

Conclusions and Future Studies

Because early diagnosis is beneficial to the curable resection of CCA patients, serum markers which can identify CCA patients at an early or non-metastatic stage are needed to be urgently explored. Identification of CCA-associated markers in the sera of patients who are at risk for CCA, such as cholangitis and periductal fibrosis, is an attractive strategy for this urgent task. A long-term surveillance study on a high risk group should be established to uncover potential early markers for CCA. To date, several serum markers have been reported to be diagnostic and prognostic markers for CCA. However, individual detection of these markers still exhibit low sensitivity and low specificity for diagnosis of CCA. One limitation to be considered is the heterogeneity of CCA in nature. Therefore, a single marker cannot cover all types of CCA and will never reach high sensitivity and specificity. Using multiple markers as "serum signature" is possibly an appropriate approach to improve the diagnostic value of serum markers for CCA. In addition, a combination of serum markers with different diagnostic approaches, such as biliary ultrasonography, CT-scan, magnetic resonance imaging, is an alternative strategy to improve the diagnosis of CCA.

Research for CCA-associated markers in bio-fluids is a focus of current research. As tumor cells exhibit distinct molecular profiles, which can be detected in bio-fluids, e.g., urine, feces and peripheral blood and its components (circulating cells, plasma, and serum), those materials allow us a noninvasive access with large quantities of samples available for analysis. Several biomarkers, such as methylated DNA, overexpressed mRNA, miRNA, and auto-antibodies, or proteins that are potentially released into the extracellular microenvironment and bio-fluids, may act as disease-specific molecular markers. Hopefully, as biomarkers are developed, they will assist in identifying those individuals without malignancy as well as assist in determining individuals who have malignancy which is amenable to surgical resection.

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